

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
19 April 2001 (19.04.2001)

PCT

(10) International Publication Number  
WO 01/26627 A1

(51) International Patent Classification<sup>7</sup>: A61K 9/127,  
33/38

(21) International Application Number: PCT/CA00/01199

(22) International Filing Date: 13 October 2000 (13.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/159,427 14 October 1999 (14.10.1999) US

(71) Applicant (for all designated States except US): 1170535  
ONTARIO INC. [CA/CA]; Suite 3000, Royal Bank Plaza,  
South Tower, 200 Bay Street, Toronto, Ontario M5J 2J1  
(CA).

(72) Inventors; and

(75) Inventors/Applicants (for US only): DICOSMO, Frank  
[CA/CA]; 4 Redinn Court, Richmond Hill, Ontario L4C

7S4 (CA). BURROWS, Lori [CA/CA]; 1299 Griffith  
Place, Oakville, Ontario L6H 2V8 (CA). DITIZIO,  
Valerio [CA/CA]; 43 Milady Road, Toronto, Ontario M9L  
2H9 (CA).

(74) Agent: BARTOSZEWICZ, Lola, A.; Sim & McBurney,  
330 Univeristy Avenue, 6th Floor, Toronto, Ontario M5G  
1R7 (CA).

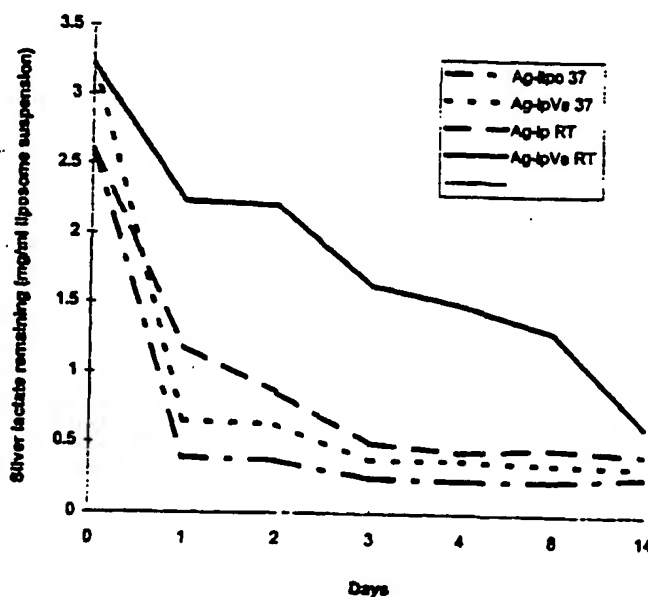
(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: LIPOSOME ENCAPSULATED SILVER SALT COMPOSITIONS

Silver Liposome Efflux



WO 01/26627 A1

(57) Abstract: The invention provides encapsulated compositions of silver salts for the treatment of infection at surface and internal wounds and other sites prone to bacterial and fungal infection. The compositions comprise liposome encapsulated silver lactate or other suitable silver salts in a pharmaceutically acceptable carrier.

**Published:**

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

--

## Liposome Encapsulated Silver Salt Compositions

### Field of the Invention

The present invention relates to encapsulated silver salt compositions for the treatment of bacterial and fungal infections in mammals. More specifically, the present invention provides liposome encapsulated silver salt compositions for treating bacterial and fungal infections in man and other mammals.

### Background of the Invention

Silver is known to have general antimicrobial properties directed against a wide range of bacteria and as such, has been used to treat infections. Silver has been used for several years in clinical settings during which time its efficacy and safety have been well-established. Clinically, silver has been used to coat the surface of polymer sheets used as wound dressings to prevent the infection of burns (Tredgett, et al., 1998).

U.S. Patent No. 1,761,590 discloses the use of silver sulfadiazine in ointments for the treatment of burns. U.S. Patent No. 4,404,197 (the disclosure of which is incorporated herein by reference) discloses the use of ointments for burns containing both silver and antibiotics, specifically norfloxacin, in which the antibiotic produces a synergistic increase in the biocidal activity of the silver. U.S. Patent No. 5,374,432 (the disclosure of which is incorporated herein by reference) discloses the use of both silver salts and antibiotics selected from aminoglycosides and quinolones, in combination, for the treatment of burns, ocular and other infections.

The encapsulation of silver sulfadiazine (SSD) in liposomes has been suggested (Lichtenstein and Margalit 1995) for the topical localized therapy of infected wounds and burns. However, no SSD encapsulated liposomes were prepared and actually used in any bioadhesive system for local targeting. The encapsulation efficiency of the process was focused upon.

Composite particles of liposomes loaded with silver or gold ultrafine particles have been prepared by UV irradiation (Sato et al 1997). Such particles were prepared to demonstrate the usefulness of liposomes as a matrix for loading and stabilizing certain ultrafine particles.

Certain bacteria are known to be particularly infectious and more resistant to killing by standard antimicrobial preparations. In particular, *Burkholderia* (formerly

*Pseudomonas) cepacia*, a common soil- and water-borne Gram negative bacterium, is known to infect patients with the genetic diseases chronic granulomatous disease (CGD) and cystic fibrosis (CF) (Govan and Deretic, 1996). *B. cepacia* is highly resistant to killing by cationic antimicrobials such as aminoglycosides, polymyxin B, and defensins due to the low levels of negatively-charged phosphate residues in the core region of its outer membrane. This property allows it to readily infect CGD patients, whose neutrophils lack the capacity to generate bacteriocidal oxygen radicals and rely only on non-oxidative cationic-peptide-based bacteriocidal mechanisms (Speert et al., 1994).

The reasons underlying the propensity of CF patients to become infected with *B. cepacia* are less clear. CF patients have mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR), a chloride channel required for proper transmembrane sodium balance (Zielenski and Tsui, 1995). The symptoms of CF include production of extremely dehydrated and viscous mucous in the pancreatic and bile ducts leading to digestive disorders, disorders of the reproductive tract ducts which often leads to infertility, and in the lungs which leads to breathing disorders, infection and finally death. The viscosity of CF pulmonary mucous impairs normal mucociliary clearance of potential pathogens, permitting some species of bacteria to establish chronic infections.

For reasons that are not completely understood, the bacterial species most likely to establish chronic pulmonary infections are *P. aeruginosa* (up to 80% of CF patients) and *B. cepacia* (10-40%, depending on the CF center). Once patients become colonized with the bacteria, they undergo repeated cycles of acute infection which lead to pulmonary damage and eventual loss of lung function. Twenty percent of patients infected with *B. cepacia* are also in danger of succumbing to "cepacia syndrome", a fulminant pneumonia and bacteremia that is rapidly fatal, often in less than 48 hours (Govan and Deretic, 1996). In addition in its resistance to cationic antibiotics, *B. cepacia* is also highly resistant to most other antibiotics (Govan et al, 1996), necessitating the use of near-toxic doses for therapy.

CF patients with extensive lung damage eventually require lung transplantation (Zuckerman and Kotloff, 1998). Most transplant centers now refuse to perform this procedure on *B. cepacia* infected patients, since the 5-year survival rates of such patients is only approximately 30%, compared with approximately 80% for patients previously colonized by *P. aeruginosa*. (Dr. S. Keshavjee, Toronto Hospital).

The inability to de-colonize the upper respiratory tract and sinuses leads to rapid re-colonization of the donor lungs with the same strain, exacerbated by patient immunosuppression that is required to prevent rejection of the transplanted tissues. Toronto General Hospital (Toronto, Canada) is one of the few remaining North  
5 American transplant centers that still accepts *B. cepacia*-infected CF patients as lung transplant candidates. However, the significant mortality rates have prompted a search for novel compounds and methods of treatment that can be used to treat or prevent *B. cepacia* infections in transplant patients.

While the bactericidal effects of silver and silver ointment compositions is  
10 known and it is also known it is possible to encapsulate SSD in a liposome, the Applicant has now unexpectedly demonstrated that the encapsulation of silver salt compositions within liposomes, in general, greatly enhances the bactericidal efficacy of silver towards bacteria. Moreover, the Applicant has now found that silver salt  
15 compositions encapsulated within liposomes can be used to treat systemic bacterial and fungal infections in mammals. This provides a novel composition and method for the treatment of patients having various infections, such as *B. cepacia*-infected CF patients.

### Summary of the Invention

20 The present invention provides encapsulated silver compositions for use in the treatment of infection in humans and in other mammals.

According to the present invention there is provided in one aspect, encapsulated silver salt compositions.

According to an aspect of the present invention there is provided a bactericidal  
25 composition, the composition comprising;

- an encapsulated silver component; and
- a pharmaceutically acceptable carrier.

According to a further aspect of the present invention there is provided an anti-fungal composition, the composition comprising;

- 30
- an encapsulated silver component; and
  - a pharmaceutically acceptable carrier.

According to yet a further aspect of the present invention is a bactericidal composition comprising an encapsulated silver component and a pharmaceutically

acceptable carrier, wherein said encapsulated silver component has a modification allowing for the targeting of such composition to specific cells and/or tissues.

According to another aspect of the present invention is the use of encapsulated silver compositions for the treatment of infection in humans and in other mammals.

5 The type of encapsulation may be selected from liposomes, microspheres, nanospheres and other lipid, polymer or protein material which may be further modified to target specific tissues and/or organs.

The compositions of the present invention may be applied topically, by injection, by inhalation or by systemic application to burn wounds, full thickness  
10 wounds, ocular infections and other infected and infection-prone areas, including internal infections of organs such as the lungs, prostate, bladder, etc.

The compositions of the present invention have use in treating a variety of bacterial infections caused by *Burkholderia cepacia* or any gram negative and gram positive bacteria in general as well as a variety of yeast infections caused by  
15 *aspergillus, fusarium, candida, phycomyces* and *allscheria*.

According to yet another aspect of the present invention is a method for treating an infection in a mammal, said method comprising the steps of administering an effective amount of an encapsulated silver salt composition in a mixture with a pharmaceutically acceptable diluent or carrier to said mammal at the site of infection.

20 According to a further aspect of the present invention is a method for treating *Burkholderia cepacia* infections in patients having chronic granulomatous disease or cystic fibrosis, said method comprising administering an effective amount of a composition comprising an encapsulated silver salt and a pharmaceutically acceptable diluent or carrier to such patients.

25 According to yet a further aspect of the present invention is a liposome composition comprising a silver salt that is stable *in vivo* and *in vitro*.

According to still a further aspect of the invention is a liposome composition having an encapsulated silver salt composition that has a long blood circulation life time.

30 The invention has particular use as an aerosol bactericidal liposome encapsulated silver lactate composition for use in combating *Burkholderia cepacia* infection in the lungs in cystic fibrosis patients.

**Brief Description of the Drawings**

A description of the preferred embodiments are provided herein below with reference to the following drawings in which:

- 5        Figure 1 shows silver liposome efflux from liposome formulations prepared containing silver lactate and/or vitamin E.

10        In the drawings, preferred embodiments of the invention are illustrated by way of example. It is to be expressly understood that the description and drawings are only for the purpose of illustration and as an aid to understanding and are not intended as a definition of the limits of the invention.

**Detailed Description of the Preferred Embodiments**

15        The compositions of the invention comprise at least one silver component provided as a solution or emulsion contained within phospholipid vesicles called liposomes. The liposomes may be unilamellar or multilamellar and are formed of constituents selected from phosphatidylcholine, dipalmitoylphosphatidylcholine, sphingomyelin, cholesterol, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, demyristoylphosphatidylcholine and combinations thereof.

20        In one embodiment, the invention includes the use of liposomes having a bilayer membrane formed of a cationic vesicle-forming lipid and a neutral vesicle-forming lipid. The liposomes have a control core with an inner surface and entrapped in the core and localized predominantly on the inner surface is the silver component.

25        The cationic lipid may include for example but are not limited to 1,2-dioleoyloxy-3-(trimethylamino) propane, N-[1-(2,3,-ditetradecyloxy)propyl]-N,N-dimethyl-N-hydroxyethylammonium bromide and dimethyldioctadecylammonium. The neutral vesicle-forming lipid is in one embodiment, a phospholipid. In a further embodiment, the neutral lipid is derivitized with a hydrophilic polymer such as polyethylene glycol.

30        The multilamellar liposomes comprise multilamellar vesicles of similar composition to unilamellar vesicles, but are prepared so as to result in a plurality of compartments in which the silver component in solution or emulsion is entrapped. Additionally, other adjuvants and modifiers may be included in the liposomal formulation such as polyethyleneglycol, or other materials such as vitamin E and



other antioxidants known to those skilled in the art which may act to stabilize the silver component incorporated therein.

While a suitable formulation of liposome includes dipalmitoyl-phosphatidylcholine:cholesterol (1:1) it is understood by those skilled in the art that  
5 any number of liposome bilayer compositions can be used in the composition of the present invention. Liposomes may be prepared by a variety of known methods such as those disclosed in U.S. Patent No. 4,235,871 (the contents of which is incorporated herein by reference) and in RRC, Liposomes: A Practical Approach. IRL Press, Oxford, 1990, pages 33-101.

10 The liposomes containing the silver component may have modifications such as having non-polymer molecules, ligands or moieties bound to the exterior of the liposome such as haptens, enzymes, antibodies or antibody fragments, cytokines and hormones and other small proteins, carbohydrates, lectins, magnetic particles (eg. aqueous-based ferro fluid EMB807), polypeptides or non-protein molecules which  
15 confer a desired enzymatic or surface recognition feature to the liposome. Surface molecules which preferentially target the liposome to specific organs tissue regions or cell types may include for example antibodies which target the liposomes to cells bearing specific antigens. Techniques for coupling such molecules are well known to those skilled in the art (see for example U.S. Patent 4,762,915 the disclosure of which  
20 is incorporated herein by reference). Alternatively, or in conjunction, one skilled in the art would understand that any number of lipids bearing a positive or negative net charge may be used to alter the surface charge or surface charge density of the liposome membrane.

The liposomes can also incorporate thermal sensitive or pH sensitive lipids as  
25 a component of the lipid bilayer to provide controlled degradation of the lipid vesicle membrane.

For systemic application by intravenous delivery it is beneficial to encapsulate the silver component within sterically-stabilized liposomes which exhibit prolonged circulation time in blood. The sterically stabilized liposomes are produced to contain  
30 polyethylene glycol as an essential component of their surface and the method of making such liposomes is well known to those skilled in the art. Furthermore, other suitable hydrophilic polymers such as polylactic acid, polyglycolic acid, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxyl propyl methacrylamide, polymethacrylamide and derivatized celluloses, such as hydroxy

methycellulose or hydroxyethylcellulose may also be used to surface coat the liposomes to effectively extend the blood circulation time of the liposomes.

The size of the liposomes can be prepared based on the intended target and route of administration. Liposomes of less than about 300 nm and preferably  
5 between about 50 nm to 300 nm may be suitable. Furthermore, the composition of the present invention may include liposomes of different sizes.

While liposomes are the preferred encapsulant for use in the composition of the present invention it is understood by those skilled in the art that other types of encapsulants may also be used to encapsulate the silver component. Microspheres  
10 including but not limited to those composed of ion-exchange resins, crystalline ceramics, biocompatible glass, latex and dispersed particles are suitable for use in the present invention. Similarly, nanospheres and other lipid, polymer or protein materials can also be used.

The silver component of the invention may be elemental silver or a silver salt.  
15 Suitable silver salts include silver acetate, silver benzoate, silver chloride, silver carbonate, silver iodate, silver iodide, silver lactate, silver laurate, silver nitrate, silver oxide, silver palmitate, silver protein, silver sulfadiazine and mixtures thereof. The preferred silver component for use in the composition of the present invention is silver lactate. The silver component will in general be incorporated in the liposome in  
20 amounts of 1-200 mM, most preferably 25-100 mM.

It is understood by those skilled in the art that the liposomes containing a silver salt may additionally contain other suitable emulsifiers and/or buffers as required. It is also understood that the liposomes may additionally comprise further active agents together with the silver salt which may help to benefit the patient. Other  
25 active agents may include but are not limited to therapeutic drugs and pharmacologically active agents such as antineoplastics, antitumor agents, antibiotics, antifungals, antivirals, antiparasitic compounds, anesthetics, cytokines and antiinflammatory compounds.

Suitable carriers for use in the present invention include but are not limited to  
30 sterile distilled water, physiological phosphate buffered saline, physiological saline, and other carriers generally regarded by current medical practice.

The compositions of the present invention provide desirable properties that make them particularly suitable for the treatment of bacterial and fungal infections that are either localized and/or systemic and of open full thickness wounds, burns

wounds, ocular infections and infections of internal organs, such as the lungs, bladder, prostate, etc. The infection-prone sites are envisaged to be any area exposed to bacteria and fungi such as full thickness wounds of the skin, scrapes, cuts, punctures and burns of the skin, as well as wounds created during surgery, the sites of

5 penetration into the body of in-dwelling devices such as catheters, drains, sutures, pins, and the like, but are not limited to these. Infections of internal organs caused by bacteria and fungi are likewise considered to be accessible to the liposome encapsulated silver salt compositions of the present invention.

The compositions of the invention provide a wide antimicrobial spectrum, as

10 is generally known to those skilled in the art, but also exhibit enhanced efficacy such that lower doses of the active agent (i.e. silver component) can be employed. This is especially beneficial since lower doses of silver reduce the risk of side-effects in the patient. Furthermore, the use of liposomes minimizes any adverse effects of the agents that they encapsulate, increase the residency of the active agent at the desired

15 site, and increase the stability of the active agent at the desired site.

In one embodiment of the present invention a pharmaceutical composition for administration to subjects in a biologically compatible form suitable for administration *in vivo* for treating various bacterial and fungal infections comprises a safe and effective amount of the encapsulated silver salt composition alone, or in

20 combination with other agents and pharmaceutical carriers. The composition may be administered to any living organism including humans and animals. By safe and effective as used herein is meant providing sufficient potency in order to decrease, prevent, ameliorate or treat a bacterial or fungal infection affecting a subject while avoiding serious side effects. A safe and effective amount will vary depending on the

25 age of the subject, the physical condition of the subject being treated, the severity of the infection, the duration of treatment and the nature of any concurrent therapy.

Administration of a therapeutically active amount of the liposome encapsulated silver salt composition of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result.

30 This may also vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability of the encapsulated silver salt composition to elicit a desired response in the subject. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be

administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

By pharmaceutically acceptable carrier as used herein is meant one or more compatible solid or liquid delivery systems. Some examples of pharmaceutically acceptable carriers are sugars, starches, cellulose and its derivatives, powdered tragacanth, malt, gelatin, collagen, talc, stearic acids, magnesium stearate, calcium sulfate, vegetable oils, polyols, agar, alginic acids, pyrogen-free water, isotonic saline, phosphate buffer, and other suitable non-toxic substances used in pharmaceutical formulations. Other excipients such as wetting agents and lubricants, tableting agents, stabilizers, anti-oxidants and preservatives are also contemplated.

The compositions described herein can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable carrier. Suitable carriers are described for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis the compositions include, albeit not exclusively, solutions of the substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The liposome encapsulated silver salt composition can also be administered parenterally or intraperitoneally. Suspensions of the active compound (the encapsulated silver salt) as a free base or pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. Dispersion can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The composition may be administered in a convenient manner such as by injection (subcutaneous, intravenous etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound of the composition from any conditions which may inactivate the compound. For oral administration, the liposomal encapsulated silver salt compositions can be delivered in the form of tablets, capsules, cachets, gelcaps, solutions, suspensions and the like.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersion and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists.

5       The liposome encapsulated silver salt composition of the present invention may be administered to a mammal alone or in combination with pharmaceutically acceptable carriers, as noted above, the proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice.

10       The silver salt liposomes of the present invention are reliably produced and effectively allow the efflux of the silver salt component contained therein (Figure 1). Furthermore, as also seen in Figure 1, the addition of antioxidants such as vitamin E for example, may act to help the encapsulation of additional silver salt within the liposomes such that more is present for efflux and thus local or systemic treatment of  
15       various infection and fungal infection. The liposome encapsulated silver salt composition of the present invention effectively inhibits the growth and spread of various species of bacteria and yeast as is shown in the following examples and therefore have use in the treatment of mammalian localized and systemic infection.

20

## Examples

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

- 5        Methods of chemistry, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

### Example 1 – General Production of Liposomes

- 10        Liposomes were made composed of dipalmitoyl-phosphatidylcholine (DPPC)/Cholesterol [1:1( %)], however, any number of lipid-to-other constituents ratio may be used to effectively achieve the embodiments of this invention. The lipids were dissolved in chloroform (4 mL) and the solvent was removed *in vacuo*. The resulting lipid film was dried *in vacuo* for two hours and rehydrated with 1 mL of  
15        1-200 mM silver lactate solutions, preferably 100 mM aqueous silver lactate solution at 45° C. Liposomes were then frozen in liquid nitrogen and thawed in a 45° C water bath, 5 times, followed by high pressure extrusion through two 100 nm –pore diameter membranes, 10 times.

- 20        This procedure produced unilamellar liposomes with an average diameter of 100 nm exhibiting equal solute distributions between the interior and exterior and yielded liposomes containing 100 mM silver lactate solution.

### Example 2 - *In Vitro* Anti-bacterial Activity against *Burkholderia cepacia* using liposomes of Example 1

- 25        A broth microdilution assay (National Committee for Clinical Laboratory Standards Protocol M100-S9) was performed to assess the minimum inhibitory concentration (MIC) of free silver lactate vs. liposomally-encapsulated silver lactate. Bacteria were grown overnight in Mueller Hintoni broth (Difco) and diluted in the same medium to match the 0.5 MacFarland turbidity standard. Empty liposomes,  
30        liposomal silver lactate (50 mM) and silver lactate (50 mM) were used undiluted and at serial doubling dilutions in Mueller-Hinton broth. One hundred µl of each compound or diluted compound was mixed with 100 µl of standardized bacterial suspension in individual wells of a polystyrene 96-well microtitre plate. The plate was

incubated in the dark in a humidified container at 37°C for 18 hr. The highest dilution that prevented growth of the bacteria (as determined by visible turbidity) was designated the MIC.

- 5 Table 1. Minimum inhibitory concentrations of silver lactate formulations.

Formulation	MIC (µg/ml) strain CIBR-C	MIC(µg/ml)- strain
<u>CIBR-L</u>		
empty liposomes	>10 mg/ml	>10 mg/ml
50 mM silver lactate	19.5 µg/ml	19.5 µg/ml
10 liposomally-encapsulated 50 mM silver lactate	9.8 µg/ml	9.8 µg/ml

### Example 3 – Additional Synthesis of Silver Lactate Encapsulated Liposomes

- 15 Dipalmitoylphosphatidylcholine (DPPC: 250 mg), cholesterol (131.5 mg) and Vitamin E ( $V_E$  1.4 mg, DPPC/ $V_E$  = 100/5) are dissolved in 5 ml chloroform. The solution was rotaevaporated for 3 hrs at 30°C. Subsequently, 5 ml of silver lactate (150mM) solution was added to the dried lipid film. The suspension was warmed to 40°C to yield a homogeneous suspension. The mixture was freeze/thawed between
- 20 liquid nitrogen and a 40°C water bath five times for 2-3 minute intervals. Subsequently, the lipid mixture was extruded three times through a 100 nm membrane filter to yield the liposomes containing silver lactate.

### Silver Lactate-loaded Liposome Characteristics:

- 25 The silver lactate-loaded liposomes are a cream or yellowish cream suspension, soluble in a mixture of chloroform/methanol/water (2.8/5.7/2) called the single phase and have a density of 1.0042.

### Silver Ion Concentration Assay:

- 30 Approximately 60µl of the silver liposome suspension (LS-Ag) was taken to which 2 drops of concentrated nitric acid and 1 ml of 2 M sulfuric acid were added. After vortexing, 3.9 ml of the single phase (see above) was added and then vortexed again. About 4 mls of chloroform, a drop of 0.04% dithizone were added and the mixture shaken vigorously until the chloroform layer is light yellow- green. The

chloroform layer was then transferred into a 10 ml volumetric flask and filled to mark with chloroform. The colour absorbance was measured at 472nm.

Reliability:

5    Linearity Test:  $R=0.9988$  ( $n=3$ )

Standard Deviation:

Stdev: 0.04509 (within day,  $n=4$ )

Stdev: 0.06898 (between day,  $n=4$ )

10    Silver Lactate Efflux Determination:

All samples in serum: phosphate buffered saline (1:1) and kept in Nalgene containers under the following conditions:

- A. Silver Lactate loaded liposomes (Ag-lipo 37) at 37° C
- B. Silver Lactate loaded Vitamin E liposome (Ag - lpVe) at 37° C
- 15    C. Liposome -Ag at room temperature
- D. Liposome-Ag with  $V_E$  at room temperature

Determination of Efflux:

20    For the Sephadex column preparation approximately 0.11 g of Sephadex G-50 was placed in a column to which 5 ml of distilled water was added with subsequent standing overnight. The aqueous phase was removed by centrifugation. About 80  $\mu$ l of the liposome samples were layered on top of the Sephadex and after 5 minutes, the column was centrifuged and the eluate was collected for determination of silver content.

25    Figure 1 shows silver lactate efflux from two liposome formulations in a 50% serum solution at room temperature and at 37 °C with and without Vitamin E ( $V_E$ ) as described above. The liposome formulations containing Vitamin E (Ag-lpVe RT and Ag-lpVe 37) encapsulate approximately 3.2 mg Silver lactate/ml of liposome suspension while the liposome formulations without Vitamin E (Ag-lipo 37 and Ag-lpRT) hold 2.6 mg Silver lactate/ml of liposome suspension. When incubated at 37  
30    °C, the Vitamin E containing liposomes show that approximately 1.2 mg silver lactate/ml of liposome suspension is retained after 24 hours and at room temperature, the liposomes retain 2.2 mg Silver lactate/ml of liposome suspension. Within the first 3 days, the amount of silver lactate remaining approached 0.5 mg silver lactate/ml of



liposome suspension at 37 °C and thereafter there was no significant change up to 14 days. When incubated at room temperature the Vitamin E containing liposomes lost approximately 50% of their load within the first three days; at 14 days 0.63 mg silver lactate/ml of liposome suspension was retained by the liposomes.

- 5 In the absence of Vitamin E (Ag-lp), liposomes retain less mg silver lactate/ml of liposome suspension than the Vitamin E containing formulation as shown in the attached graph. At 37 °C and at room temperature, liposomes without Vitamin E tended to efflux their load of silver lactate more rapidly than liposomes with Vitamin E. The most rapid efflux occurred at 37 °C, as expected; approximately 0.4 and 0.3  
10 mg silver lactate/ml of liposome suspension was retained after 14 days were retained when incubated at room temperature and 37°C, respectively.

- The most stable liposome formulation in terms of silver lactate retention was that containing Vitamin E (Ag-lp Ve). The Vitamin E serves to stabilize the liposome system from apparent oxidation and is therefore beneficial for the formulation. While  
15 it is recommended that the silver lactate containing liposome formulations of the present invention incorporate Vitamin E or other suitable antioxidant it is not required.

#### Reliability and Reproducibility

- 20 Linearity Test:  $R=0.9999$  ( $n=3$ ) Cf. The chart for linearity test.  
Standard Deviation:  
Stdev: 0.012302 (within day,  $n=4$ )  
Stdev: 0.027667 (between day,  $n=3$ )  
FL =  $1.71 \pm 0.02954$  ( $P = 0.95$ )  
25 FL% = 1.726% ( $P = 0.95$ )

**Example 4: Minimal inhibitory concentration values for liposomal silver lactate**

Species	MIC ( $\mu\text{g/ml}$ )
<i>Acinetobacter baumannii</i>	6.3
<i>Burkholderia cepacia</i>	6.3
<i>Candida albicans</i>	6.3
<i>Escherichia coli</i>	6.3
<i>Klebsiella pneumoniae</i>	12.5
<i>Pseudomonas aeruginosa</i>	6.3
<i>Staphylococcus aureus</i>	6.3
<i>Staphylococcus epidermidis</i>	3.1
<i>Sienotrophomonas maltophilia</i>	6.3

- 5 Bacteria were grown from glycerol stocks overnight at 37°C in Mueller Hinton broth. Cells were harvested by centrifugation and washed three times in sterile phosphate buffered saline, pH 7.0. The culture densities were adjusted to match a MacFarland standard of 0.5 (approximately  $10^7$  colony forming units per ml). Liposomes loaded with silver lactate (4.0 mg/ml) were diluted in Mueller-Hinton
- 10 broth to 50  $\mu\text{g/ml}$  silver lactate. Doubling dilutions of 100  $\mu\text{l}$  were set up across the columns of 2 polystyrene 96-well microtitre plates. Each species was tested across a single row (10  $\mu\text{l}$  added per well), with Mueller Hinton alone inoculated as a positive control for growth and uninoculated Mueller Hinton as a negative control. The plates were incubated overnight at 37°C in a humidified chamber. The MIC was scored as
- 15 the highest dilution preventing growth of the organism.

### References

- 5 Tredgett, E.E., Shankowsky, H.A., Groeneveld, M.N., and Burrell, R. 1998. A matched-pair, randomized study evaluating the efficacy and safety of ACTICOAT silver-coated dressing for the treatment of burn wounds. *J. Burn Care & Rehab.* 19: 531-537.
- 10 Govan JR., Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomona~ aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev.* 1996 Sep;60(3):539-74.
- 15 Lichtenstein A., and Margalit, R. Liposome-Encapsulated Silver Sulfadiazine (SSD) for the Topical Treatment of Infected Burns: Thermodynamics of Drug Encapsulation and Kinetics of Drug Release. *Journal of Inorganic Biochemistry*, 60, 187-198 (1995).
- 20 Speert DP, Bond M, Woodnian RC, Curnutte T. Infection with *Pseudomonas cepacia* in chronic granulomatous disease: role of nonoxidative killing by neutrophils in host defense. *J Infect Dis.* 1994 Dec;170(6):1524-31.
- Zielenski .1, Tsui LC. Cystic fibrosis: genotypic and phenotypic variations. *Annu Rev Genet.* 1995; 29:777-807.
- 25 Govan JR., Hughes JE, Vandamme P. *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J Med Microbiol.* 1996 Dec;45(6):395-407.
- Zuckerman JB, Kotloff RM. Lung transplantation for cystic fibrosis. *Clin Chest Med.* 1998 Sep;19(3):535-54, vii.

**Claims**

1. An encapsulated silver salt composition for the treatment of local and systemic bacterial and/or fungal infection.
- 5 2. The composition of claim 1, wherein said silver salt is selected from the group consisting of silver acetate, silver benzoate, silver chloride, silver carbonate, silver iodate, silver iodide, silver lactate, silver laurate, silver nitrate, silver oxide, silver palmitate, silver protein, silver sulfadiazine and mixtures thereof.
- 10 3. The composition of claim 1, wherein said encapsulation is within an encapsulant selected from the group consisting of liposome, microsphere and nanosphere.
- 15 4. The composition of claim 3, wherein said encapsulant is modified to provide surface recognition to target a specific body tissue or cell type.
- 20 5. The composition of claim 4, wherein said modification is selected from the group consisting of haptens, enzymes, antibodies, antibody fragments, cytokines, hormones, carbohydrates, lectins, magnetic particles, polypeptides, non-protein molecules and combinations thereof.
- 25 6. The composition of claim 1, wherein said composition additionally comprises an agent selected from the group consisting of an antioxidant, an emulsifier and a buffer.
- 30 7. The composition of claim 1, wherein said composition is provided within a suitable carrier.
8. The composition of claim 1, wherein said composition additionally comprises an active agent selected from the group consisting of antineoplastic agents, antitumor agents, antibiotics, antifungals, antivirals, antiparasitic compounds, anesthetics, cytokines and antiinflammatory compounds.

9. The composition of claim 1, wherein said composition is effective against infection by *Acinetobacter baumannii*, *Burkholderia cepacia*, *Candida albicans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Asperillus*, *Fusarium*, *Phycomyces*, *Allscheria*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Stenotrophomonas maltophilia*.
10. A bactericidal and anti-fungal composition, the composition comprising;
- an encapsulated silver component; and
  - a pharmaceutically acceptable carrier.
11. The use of an encapsulated silver salt composition for the treatment of *Burkholderia Cepacia* in genetically diseased patients.
12. The use of claim 11, wherein said encapsulation is an encapsulant selected from the group consisting of liposomes, microspheres and nanospheres.
13. The use of claim 11, wherein said genetic disease is selected from the group consisting of chronic granulomatous disease and cystic fibrosis.
14. A method for treating an infection in a patient, said method comprising the steps of administering an effective amount of an encapsulated silver salt composition in a mixture with a pharmaceutically acceptable diluent or carrier to said patient at the site of infection.
15. The method of claim 13, wherein said method is used for treating *Burkholderia cepacia* infections in patients having chronic granulomatous disease or cystic fibrosis.
16. The method of claim 14, wherein the silver salt composition is encapsulated in vehicle selected from the group consisting of liposomes, microspheres and nanospheres.

1/1

## Silver Liposome Efflux

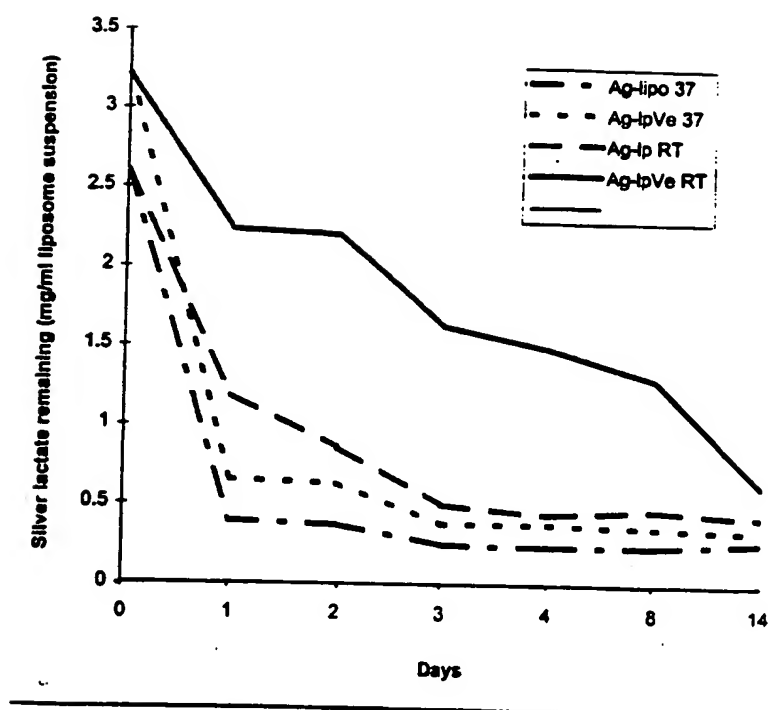


FIGURE 1

# INTERNATIONAL SEARCH REPORT

National Application No.

PCT/CA 00/01199

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 A61K9/127 A61K33/38

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 138 740 A (ENQUAY) 24 April 1985 (1985-04-24) claims 1-4,6,9-11,16,17,24-40 page 26, line 20 - line 21 page 45, line 35 - line 37 examples 12,13	1-3, 6-10,12
X	EP 0 355 009 A (K.MINNINGER) 21 February 1990 (1990-02-21) claims examples	1-3,6-8
X	US 4 391 799 A (A.D.MASON ET AL.) 5 July 1983 (1983-07-05) claims examples	1-3, 7-10,14
	--- -/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*A\* document member of the same patent family

Date of the actual completion of the international search

12 March 2001

Date of making of the international search report

19/03/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV/Riswijk  
Tel: (+31-70)340-2040 NTX: 31 651-repoint.  
Fax: (+31-70)340-3016

Authorized officer

Scarponi, U

# INTERNATIONAL SEARCH REPORT

national Application No  
PCT/CA 00/01199

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 08470 A (GILTECH) 9 August 1990 (1990-08-09) --  claims page 7, line 12 - line 31 page 10, line 20 - line 24	1-3, 7-10,14, 16
X	WO 93 19735 A (CNRS,FR) 14 October 1993 (1993-10-14) claims 1,3-5,10,12 page 12, line 20 - line 22 page 13, line 9 page 13, line 11 - line 18 page 14, line 8 - line 25 examples 1,3-6,8-10	1-3,6,7
X	GB 929 406 A (UPJOHN) 19 June 1963 (1963-06-19) claims example 6 page 2, line 100 page 2, line 118	1-3,6,7



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 00/01199

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 138740	A	24-04-1985	US 4563184 A	07-01-1986
			AT 61233 T	15-03-1991
			AU 563517 B	09-07-1987
			AU 3362384 A	26-04-1985
			CA 1245158 A	22-11-1988
			DE 3484222 D	11-04-1991
			EP 0344090 A	29-11-1989
			ES 536779 D	01-04-1987
			ES 8704345 A	16-06-1987
			IL 73188 A	12-07-1990
			JP 1853380 C	07-07-1994
			JP 5066147 B	21-09-1993
			JP 60150755 A	08-08-1985
			KR 9205822 B	20-07-1992
			PH 20290 A	18-11-1986
			US 4857334 A	15-08-1989
			US 4820292 A	11-04-1989
			US 4725271 A	16-02-1988
			US 4747845 A	31-05-1988
			ZA 8407592 A	29-05-1985
EP 355009	A	21-02-1990	DE 3828044 A	10-08-1989
			AU 2936489 A	25-08-1989
			AU 4045489 A	23-03-1990
			DK 94090 A	13-06-1990
			WO 8906962 A	10-08-1989
			WO 9001934 A	08-03-1990
			EP 0326145 A	02-08-1989
			PT 91481 A	08-03-1990
US 4391799	A	05-07-1983	NONE	
WO 9008470	A	09-08-1990	AT 150935 T	15-04-1997
			DE 69030374 D	07-05-1997
			DE 69030374 T	16-10-1997
			DK 455706 T	13-10-1997
			EP 0455706 A	13-11-1991
			ES 2099708 T	01-06-1997
			JP 2989888 B	13-12-1999
			JP 4503018 T	04-06-1992
			US 5470585 A	28-11-1995
WO 9319735	A	14-10-1993	FR 2689418 A	08-10-1993
			CA 2133421 A	14-10-1993
			DE 69300823 D	21-12-1995
			DE 69300823 T	01-08-1996
			EP 0633768 A	18-01-1995
			ES 2082643 T	16-03-1996
			JP 7505330 T	15-06-1995
			US 5792472 A	11-08-1998
GB 929406	A		NONE	